DESCRIPTION

GENE ENCODING A PROTEIN HAVING A GLYCOSYL TRANSFERASE ACTIVITY TO AURONES

Technical Field

The present invention relates to a gene encoding a protein having a glycosyl transferase activity to aurones, said protein, and the uses thereof.

Background Art

The color of flowers are mainly based on three pigments: flavonoids, carotenoids, and betalains. Yellow colors are mostly derived from carotenoids and betalains, but in some plants they are derived from flavonoids. Among the flavonoid pigments, major pigments that are thought to be associated with the development of yellow flowers are divided into three groups: chalcones, aurones, and yellow flavonols (Saito, Biohorti 1, pp. 49-57, 1990)

Aurones are substances in which two phenyl groups are joined together through three carbon atoms of dihydrofuran. As aurones, there are known 4,6,4'-trihydroxy aurone, aureusidin, sulfuretin, bracteatin, and the like. For example, aureusidin and bracteatin are contained in snapdragons, aureusidin is contained in limoniums, aureusidin is contained in morning glories, sulfuretin is contained in dahlias, bracteatin is contained in Helichrysum bracteatum, and sulfuretin is contained in Helianthus tuberosus.

Flavonoids have generally been modified by acylation, glycosilation, methylation and the like, and carotenoids and betalains have also been glycosilated in many cases. Among various modifications, glycosilation plays an important role in the color of flowers such as (1) contribution to enhancing the stability and solubility of pigments, (2) the presence as a step

preceding acylation that greatly affects the hue of colors, and (3) copigmentation effects by the glycosilated flavonoids, and the like.

It has been reported that, in snapdragon, a yellow pigment aurones (aureusidin, bracteatin), a kind of flavonoid, is present in a glycosilated at its position 6 corresponding to position 7 of flavonoids, and since aurones are present as glycosides in other aurone-containing plants as well, it has been considered that glycosilation is essential for the stability of aurones.

There are many reports on the genes for glycosyl transferases derived from plants that transfer a glycosyl group to flavonoids and on the activities of those enzymes.

By way of example, genes encoding UDP-glucose: flavonoid 3-glucosyl transferases (3GT) that transfer a glycosyl group to the position 3 of flavonoids have been obtained from many plants including corn, barley, and snapdragon, and has been analyzed in detail (The Flavonoids: Advanced in Research Since 1986. Published by Chapman & Hall, 1993).

Also, genes encoding UDP-glucose: flavonoid 5-glucosyl transferases (5GT) that transfer a glycosyl group to the position 5 of flavonoids have been cloned from perillas, torenias, and verbenas (International Patent Publication No. WO 99/05287).

However, as to the gene encoding UDP-glucose: flavonoid 7-glucosyl transferase (7GT) that transfers a glycosyl group to the position 7 of flavonoids, there is only one report on the purification of flavanone-specific 7-glucosyl transferase in grapefruits (Archives of Biochemistry and Biophysics 282, 1: 50-57, 1990).

With regard to enzymes that transfer a glycosyl group to the position 6 of aurones, there is a report on the measurement of a reaction that transfers a glycosyl group to the position 6 of sulfuretin, a kind of aurone (Plant Science 122: 125-131, 1997), but this only studied

the enzymatic property using a partially purified product, and has not been purified in a pure form.

On the other hand, there is a report on the isolation of a glycosyl transferase, pS.b UFGT1, that has an activity of transferring glucose to the position 7 of baicaleins, a kind of flavone, from the hairy roots of a Labuatae, Scutellaria baicalensis (1997, presented at the Fifteenth annual meeting of Japanese Society of Plant Cell and Molecular Biology). The gene product is also reported to be capable of transferring a glycosyl group to the position 7 of anthocyanidins and flavonols, but not reported on aurones (presented at the Fifteenth annual meeting of Japanese Society of Plant Cell and Molecular Biology).

As genes having a high homology to pS.b UFGT1, tabacco-derived IS10a and IS5a have been reported (Plant Molecular Biology, 31: 1061-1072, 1996), but its activity of transferring a glycosyl group to position 7 (7GT activity) has not been studied.

Reports to date teach that the glycosyl transferases that use flavonoids as substrates have a great variation in substrate specificity even among flavonoids. For example, when the gene of flavonoid-3-glycosyl transferase derived from gentians were cloned, expressed in E. coli, and the activity was determined, it was found to exhibit a 61% activity to cyanidins, a 38% activity to pelargonidins, and a good activity to anthocyanins relative to a 100% glycosyl transferase activity to delphinidins. On the other hand, it only shows an activity of 7.0%, 6.5%, and 4.4% to kaempferol, quercetin, and myricetin, respectively. Furthermore, it does not transfer a glycosyl group to dihydroflavonols (Tanaka et al., Plant Cell Physiol. 37: 711, 1996).

Also, when the gene of flavonoid-3-glycosyl transferase derived from grapes was cloned and the activity was determined in E. coli, its Km was 30 μM and Vmax was 905 nkatals/mg to cyanidins, whereas to

quercetins the Km was 15 μM and Vmax was 18.9 nkatals/mg, exhibiting a great difference in reaction rates (Ford et al., J. Biol. Chem. 273: 9224, 1998).

These reports indicate that glycosyl transferases can distinguish the kinds of flavonoids and that the glycosyl transferase activity to a flavonoid does not readily permit the estimation of the glycosyl transferase activity to another flavonoids.

Disclosure of the Invention

As hereinabove described, glycosyl transferases using flavonoids as substrates have a great variation in substrate specificity and the estimation of a glycosyl transferase activity to a specific flavonoid cannot be easily made based on known glycosyl transferases.

Thus, the present inventors have attempted to obtain a gene encoding a protein having a glycosyl transferase activity to aurones among the flavonoid pigments, and thereby have completed the present invention.

The present inventors have demonstrated that a gene product of the pS.b UFGT1 gene derived from Scutellaria baicalensis has an activity of transferring a glycosyl group to aurones, and, using this gene as a probe, have obtained a gene encoding a protein having an activity of transferring a glycosyl group to aurones from snapdragons (Antirrhinum majus).

Also, using said gene obtained from snapdragons (Antirrhinum majus) as a probe, the present inventors have further obtained two genes encoding a protein having an activity of transferring a glycosyl group to aurones from a petunia (Petunia hybrida).

Thus, the present invention provides a gene encoding a protein having an activity of transferring a glycosyl group to aurones. Furthermore, the present invention provides a gene encoding a protein having the amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10 and having an activity of transferring a glycosyl group to

aurones.

The present invention further provides a gene encoding a protein that has an amino acid sequence modified by the addition, deletion and/or substitution with other amino acids of one or more amino acids in the amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10, and that has an activity of transferring a glycosyl group to aurones.

The present invention further provides a gene encoding a protein that hybridizes to a nucleic acid having a nucleotide sequence encoding the amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10 or a portion thereof under a stringent condition, and that has an activity of transferring a glycosyl group to aurones.

The present invention also provides a vector comprising said gene.

The present invention further provides a host transformed with said vector. The host may be a microorganism, plant cells, animal cells, or plants.

The present invention also provides a method of producing a protein having an activity of transferring a glycosyl group to aurones, by culturing, cultivating or breeding said host.

The present invention also provides a method of stabilizing aurones in the plant, said method comprising introducing said gene into the plant having aurones, allowing said gene to be expressed, and transferring a glycosyl group to aurones in the plants with a protein thus produced.

In cases where a new flower color is to be created by introducing and expressing the gene of an aurone synthase in plants that have no aurones, aurones can be stably expressed therein by expressing the gene obtained by the present invention.

Brief Description of Drawings

Figure 1 shows a process of constructing the plasmid

pESBGT-1.

Figure 2 shows a process of constructing the plasmid pETAmGT1.

Embodiments for Carrying out the Invention

First, a cDNA library is prepared from the petals of a yellow snapdragon. The cDNA library thus obtained is screened using pS.b UFGT1, a flavonoid-7-glycosyl transferase gene derived from Scutellaria baicalensis, and then a clone is obtained. The plasmid obtained from the clone is isolated and its nucleotide sequence is determined.

It is known that enzymatically active proteins have regions essential for the enzymatic activity and regions non-essential for the activity, and that the enzymatic activity is retained even when the non-essential regions are modified by the addition, deletion and/or substitution with other amino acids of one or more amino acids. Thus, the present invention encompasses not only a protein having an amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10, but also a protein having an amino acid sequence modified by the addition, deletion and/or substitution with other amino acids of one or more amino acids in the amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10, and that having an activity of transferring a glycosyl group to aurones, and a gene encoding said protein.

The number of amino acids to be modified is, for example, 50 or less, and preferably 30 or less, for example 20 or less or 10 or less.

The gene encoding the protein having an amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10 can be obtained as cDNA or genomic DNA from snapdragons or petunias. The method of cloning cDNA is specifically described in Examples 2, 3 and 6. In order to obtain genomic DNA, a genomic library is constructed based on the standard method from snapdragons or petunias and then

screened using said cDNA or a fragment thereof according to the standard method.

A gene encoding a protein having an amino acid sequence modified in the amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10 can be constructed by modifying a nucleotide sequence of a DNA, for example cDNA, encoding a protein having an amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10, by a standard method for manipulating genes such as site-directed mutagenesis and the PCR method.

Once a gene encoding a protein having the enzymatic activity has been cloned, the nucleic acid that hybridizes to said gene or a portion thereof encodes, in most cases, an amino acid sequence that exhibits the enzymatic activity and that is similar to the original protein. Thus the present invention provides a gene that hybridizes to a nucleic acid having a nucleotide sequence encoding an amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10 or a portion thereof under a stringent condition, and that encodes a protein having an activity of transferring a glycosyl group to aurones.

In the above hybridization condition, the washing condition is preferably 5 × SSC, 0.1% SDS and 50°C, more preferably 2 × SSC, 0.1% SDS and 50°C, and more preferably 0.1 × SSC, 0.1% SDS and 50°C.

In the above hybridization, when a nucleic acid having a portion of the nucleotide sequence encoding an amino acid sequence as set forth in SEQ ID NO: 2, 8, or 10 is used, the length of the nucleic acid is preferably at least 17 base pairs long, and more preferably at least 100 base pairs long. As target nucleic acids to be hybridized, there can be used nucleic acids prepared from Scutellaria baicalensis, snapdragons, petunias, limoniums, mornig glories, dahlias, Helichrysum bracteatum, Helianthus tuberosus, and the like, and preferably genomic DNA libraries or cDNA libraries are

used.

The present invention also provides a method of producing the above protein having an activity of transferring a glycosyl group to aurones. The method comprises introducing a vector comprising DNA encoding said protein into a host, culturing or growing said host, and recovering said protein as desired. The host may be host cells or plants, etc.

As the host cells, there can be used prokaryotic cells, particularly bacteria cells such as cells of Escherichia coli, a bacterium belonging to the genus Bacillus such as Bacillus subtilis and Bacillus brevis, lower eukaryotes such as fungi, for example yeast such as a yeast belonging to the genus Saccharomyces, for example Saccharomyces cerevisiae, or filamentous fungi such as the genus Aspergillus, for example Aspergillus oryzae and Aspergillus niger, and the like.

Furthermore, as higher eukaryotic hosts, there can be mentioned insect cells such as cells of silkworm, animal cells such as CHO cells, cultured human cells such as HeLa cells, and the like.

The gene of the present invention may also be expressed in an organism of, for example, a plant and so on.

Vectors comprising the DNA of the present invention, expression vectors in particular, may contain expression regulatory regions, and the expression regulatory regions depend on the host cell. For example, as promoters for bacterial expression vectors, there can be mentioned commonly used promoters such as the trc promoter, the tac promoter, the lac promoter, the T7 promoter and the like; as promoters for yeast expression vectors, there can be used the promoters of the genes of the glycolytic pathway such as glyceraldehyde-3-phosphate dehydrogenase promoter, galactokinase promoter, and the like; and as promoters for animal cell expression vectors, viral promoters can be used.

In order to recover proteins having an activity of transferring a glycosyl group to aurones, methods commonly used for isolation and purification of protein can be used such as liquid chromatography, and affinity chromatography.

With the current state in the art, it is possible to further ligate the cDNA under the control of a constitutive or inducible promoter, and introduced into a plant such as petunia, rose, carnation, chrysanthemum, torenia, verbena, gerbera, tobacco, strawberry, lisianthus, gentian, gladiolus, and tulip in a system utilizing Agrobacterium, particle guns, or electroporation, and to express the gene encoding the protein having an activity of transferring a glycosyl group to aurones in flower petals.

It is expected that in the flower petals in which a protein having an activity of transferring a glycosyl group to aurones was expressed, the aurones are glycosilated, and thereby are stabilized. The plants thus obtained can provide flowers having a hue of color that cannot be found in the conventional varieties.

In plants having no aurones, an aurone synthase gene are introduced, expressed, and at the same time a gene of the present invention encoding the protein having an activity of transferring a glycosyl group to aurones can be introduced and expressed, so that aurones can be stably expressed and new plants having a yellow hue can be provided. As the above plants having no aurones, there can be mentioned petunias, torenias, and tobaccoes.

Examples

The present invention will now be explained in further details with reference to the following Examples.

Example 1. Measurement of the activity of transferring a glycosyl group to aurones of a product of the pS.b UFGT1 gene derived from Scutellaria baicalensis

The activity of the pS.b UFGT1 gene derived from Scutellaria baicalensis of transferring a glycosyl group to aurones was determined using an expression vector pESBGT-1 in E. coli prepared by the following method.

First, the pS.b UFGT1 gene was subjected to a PCR reaction using two primers to introduce NdeI and BamHI sites.

5'-ATA ACT ACA TAT GGG ACA ACT CCAC-3' (SEQ ID NO: 3)

5'-CAG AAC AGG ATC CAC ACG TAA TTT A-3' (SEQ ID NO: 4)

The PCR reaction mixture was prepared in a total volume of 50 μl comprising 300 ng of pSBGT-1, 1 × Native Pfu DNA polymerase reaction buffer (Stratagene), 0.2 mM dNTPs, 4 pg/μl each of the primers, and 2.5 U of Native Pfu DNA polymerase. The reaction was carried out, after 3 minutes at 95°C, for 30 cycles with one cycle comprising 95°C for 1 minute, 50°C for 2 minutes, and 72°C for 2 minutes, and finally was treated at 72°C for 7 minutes.

The PCR product was digested with NdeI and BamHI, and then was ligated to the NdeI- and BamHI-digested pET-3a vector (Stratagene) to construct pESBGT-1 (Figure 1). Using each of pESBGT-1 and pET-3a vector, it was transformed into Epicurian Coli BL21 (DE3) (Stratagene). The transformants were incubated overnight at 37°C in 3 ml of a LB medium containing 50 μg/ml of ampicillin. The preculture (500 μl) was added to 50 ml of a LB medium containing 50 μg/ml of ampicillin, and cultured until A600 reached 0.6-1.0. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added thereto to a final concentration of 0.5 mM, which was cultured at 28°C for 4 hours and centrifuged (5000 rpm, 10 minutes, 4°C) to collect the cells.

The pellets were suspended in 5 ml of the buffer (10

mM sodium phosphate, pH 6.5, 1 mM β-mercaptoethanol (2-ME)). After the E. coli cells were disrupted by a sonicator, it was centrifuged (15,000 rpm, 5 minutes, 4°C), and the supernatant obtained was used as a crude enzyme solution for the next enzyme reaction.

In addition to aureusidin, the enzymatic activity was determined using naringenin or luteolin as the substrate.

For aureusidin, the enzymatic activity was determined as follows:

To 50 µl of the crude enzyme solution were added 0.1 M Tris-HCl, pH 8.0, and 150 µl of 0.05% 2-ME, and then incubated at 30°C for 10 minutes. Then 5 µl of 4.66 mM aureusidin and 50 µl of 5 mM UDP-glucose were added thereto, and was allowed to react at 30°C for 1 hour. After the reaction was stopped by adding 200 µl of 90% acetonitrile containing 5% trifluoroacetic acid (TFA), it was centrifuged at 15,000 rpm and 4°C for 3 minutes. The supernatant thus obtained was filtered (pore size 0.45 µm, 4 mm Millex-LH, Millipore) to remove insoluble substances. The filtrate was analyzed by high performance liquid chromatography.

The analytical condition was as follows: The column used was Asahipak-ODP-50 (4.6 mm ϕ × 250 mm, Showa Denko). The mobile phase comprised water containing 0.1% TFA as solution A and 90% CH_3CN containing 0.1% TFA as solution B. After a linear gradient from 20% solution B to 100% solution B for 20 minutes, 100% solution B was retained for 5 minutes. The flow rate was 0.6 ml/min. Detection used A380 nm, and an absorption spectrum at 250-400 nm using Shimadzu PDA detector SPD-M6A.

For a reaction of the crude extract of E. coli cells in which pESBGT-1 was expressed, new substances were detected that eluted at 9.7, 12.0, and 13.1 minutes in addition to the substrate aureusidin (retention time 18.1

minutes). Since they were not detected in a reaction of the crude extract similarly prepared from E. coli cells in which the pET-3a vector was expressed, they were considered to be products resulting from the protein derived from pESBGT-1. The substance that eluted at 12.0 minutes among the products had the same retention time and the same absorption spectrum as that of aureusidin 6-glycoside. Other products also are considered to be aureusidin glycosides based on the absorption spectra.

For naringenin and luteolin, the enzymatic activity was determined as follows.

To 20 μl of the crude enzyme solution were added 25 μl of 0.1 M citric acid-phosphate buffer, pH 6.5, 5 μl each of 5 μM substrate, and 25 μl of 5 mM UDP-glucose in a total volume of 250 μl, and then incubated at 30°C for 30 minutes. After the reaction was stopped by adding 200 μl of 90% acetonitrile containing 5% TFA, it was centrifuged at 15,000 rpm and 4°C for 3 minutes. The supernatant thus obtained was filtered (pore size 0.45 μm, 4 mm Millex-LH, Millipore) to remove insoluble substances. The filtrate was analyzed by high performance liquid chromatography.

The analytical condition for naringenin was follows: The column used was YMC J's sphere ODS-M80 (4.6 mm ϕ × 150 mm, YMC). The mobile phase comprised water containing 0.1% TFA as solution A and 90% CH_3CN containing 0.1% TFA as solution B. After a linear gradient from 20% solution B to 80% solution B for 10 minutes, 80% solution B was retained for 5 minutes. The flow rate was 0.6 ml/min. Detection used A290 nm, and an absorption spectrum at 250-400 nm using Shimadzu PDA detector SPD-M6A.

The analytical condition for luteolin was as follows: The column used was YMC J's sphere ODS-M80 (4.6 mm ϕ × 150 mm, YMC). The mobile phase comprised water

containing 0.1% TFA as solution A and 90% CH_3CN containing 0.1% TFA as solution B. After a linear gradient from 20% solution B to 80% solution B for 10 minutes, 80% solution B was retained for 5 minutes. The flow rate was 0.6 ml/min. Detection used A330 nm, and an absorption spectrum at 250-400 nm using Shimadzu PDA detector SPD-M6A.

When naringenin was used as the substrate, a new substance was detected that eluted at 6.9 minutes in addition to the naringenin (retention time 9.7 minutes). The substance was not detected in a reaction of the crude extract similarly prepared from E. coli in which the pET-3a vector was expressed. It had the same retention time as naringenin 7-glycoside but a different absorption spectrum, suggesting that a plurality of naringenin glycosides are present each at a trace amount.

When luteolin was used as the substrate, new substances were detected that eluted at 6.4, 7.7, and 8.0 minutes that were not be detected in a reaction of the crude extract similarly prepared from E. coli in which the pET-3a vector was expressed. The substance that eluted at 6.4 minutes among them had the same retention time as luteolin 7-glycoside.

The above result indicated that the pS.b UFGT1 gene derived from Scutellaria baicalensis is an enzyme that can glycosilate aureusidin. It was also demonstrated that it can glycosilate luteolin but had very little effect on naringenin.

It has already been shown that baicalein can be glycosilated at the position 7. After the reaction is complete for baicalein, almost 100% is detected as a 7 glycoside, but no reaction occurred to naringenin indicating that the expression product of the Scutellaria baicalensis-derived pS.b UFGT1 gene has a high substrate specificity.

Example 2. Construction of cDNA library of snapdragon petals

A cDNA library of the petals was prepared as follows: From 5 g of fresh petals of a yellow snapdragon (yellow butterfly), RNA was obtained using a method of employing guanidine thiocyanate and cesium chloride as described in detail in Method in Molecular Biology, Vol. 2, (Humana Press Inc., 1984) by R. McGookin et al., and polyA+RNA was purified therefrom using Oligotex dT30 (Nippon Roche). From the polyA+RNA, cDNA library was constructed using the cDNA synthesis kit, Uni-XR vector kit (Stratagene). The library obtained comprised 1.6×10^5 plaque forming units (pfu).

Example 3. Collection of the full-length aurone glycosyl transferase

The snapdragon cDNA library obtained in Example 2 was screened using the full-length pS.b UFGT1, a hairy root-derived flavonoid-7-glycosyl transferase. The library was screened using a non-radio system DNA detection kit (Boehringer). Hybridization was carried out overnight at 37°C. Washing filter was carried out at 5 × SSC, 0.1% SDS and 50°C for 30 minutes. About 200,000 plaques were screened to finally obtain 2 clones. The method was based on Molecular Cloning (Sambrook et al., Cold Spring Harbour Laboratory Press, 1989).

Since the two clones encoded the sequences having the completely same length, one was designated as pAmGT1 and nucleotide sequence was determined.

The nucleotide sequence was determined by synthesizing an oligonucleotide primer and using DNA Sequencer model 310 (Applied Biosystems). The nucleotide sequence and the deduced amino acid sequence are shown in SEQ ID NO: 1 in the sequence listing.

pAmGT1 contained a 1751 bp gene AmGT1 encoding a protein of a molecular weight 53.9 kDa comprising 481 amino acids.

Example 4. Expression of the AmGT1 cDNA in E. coli

The expression of the AmGT1 cDNA was carried out

using the pET System (Stratagene).

First, in order to introduce NdeI and BamHI sites, the following 2 primers pETAmGT5' and pETAmGT3' were used in a PCR reaction.

pETAmGT5': 5'-ATA ACT ACA TAT GGG AAA ACT TCA C-3' (SEQ ID NO: 5)

pETAmGT3': 5'-GAA CAG GAT CCA CAC ACT AGA AGT CA-3' (SEQ ID NO: 6)

The PCR reaction mixture was prepared in a total volume of 100 μl comprising 100 ng of pAmGT1, the 1 × the cloned Pfu DNA polymerase reaction buffer (Stratagene), 0.2 mM dNTPs, 0.5 pmol/μl each of the primers, and 5.0 U of the cloned Pfu DNA polymerase. The reaction was carried out, after 45 seconds at 95°C, for 25 cycles with one cycle comprising 95°C for 45 seconds, 50°C for 45 seconds, and 72°C for 2 minutes, and was finally treated at 72°C for 10 minutes. The PCR product obtained was subcloned into the pCR2.1 TOPO vector (INVITROGEN).

Some of the clones of the plasmid pTOPO-ETAmGT1 thus obtained were reacted using M13 Reverse Primer and M13(-20) primer (TOYOBO) using ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and the nucleotide sequences on both ends were confirmed using DNA Sequencer model 310 (Applied Biosystems). An about 2.7 Kb fragment obtained by digesting pTOPO-ETAmGT1 with NdeI, BamHI and ScaI was ligated to the NdeI and BamHI sites of the pET-3a vector (Stratagene) to obtain plasmid pETAmGT1 (Figure 2). Using pETAmGT1, it was transformed into Epicurian Coli BL21 (DE3) (Stratagene).

Example 5. Measurement of the glycosyl transferase activity of the AmGT1 cDNA recombinant protein

The transformant obtained in Example 4 was cultured, extracted and the enzymatic activity was measured as in Example 1.

When aureusidin was used as the substrate, new

substances were detected that eluted at 10.98, 11.27, and 11.85 minutes in addition to aureusidin (retention time 16.6 minutes). Since the substances were not detected in a reaction of the crude extract similarly prepared from E. coli in which the pET-3a vector was expressed, they were believed to be products that resulted from pESBGT-1-derived protein.

Among the products, the substance that eluted at 10.98 minutes had the same retention time as aureusidin 6-glycoside, and the one that eluted at 11.85 minutes had the same retention time as aureusidin 4-glycoside.

The above results indicated that AmGT1 can transfer a glycosyl group to the positions 6 and 4 of aureusidin. The substance that eluted at 11.27 minutes is also believed to be aureusidin glycoside based on the absorption spectra.

Example 6. Preparation of the gene of aurone glycosyl transferase derived from petunias

A cDNA library obtained from petals of a petunia variety "Old Glory Blue" (Nature 366: 276-279, 1993) was screened with the full-length AmGT1 gene obtained in Example 3. The library was screened using a non-radio system DNA detection kit (Boehringer). Hybridization was carried out overnight at 37°C. Washing filter was carried out at 5 × SSC, 0.1% SDS, and 50°C for 30 minutes. About 200,000 plaques were screened to finally obtain 2 clones. The method was based on Molecular Cloning (Sambrook et al., Cold Spring Harbour Laboratory Press, 1989).

The two clones were designated as pPh7GTa and pPh7GTb, respectively, and the nucleotide sequences were determined. The nucleotide sequence was determined by synthesizing an oligonucleotide primer and using DNA Sequencer model 310 (Applied Biosystems). The nucleotide sequence at the insertion site of pPh7GTa and the deduced amino acid sequence are shown in SEQ ID NO: 7 and 8, respectively, and the nucleotide sequence at the

insertion site of pPh7GTb and the deduced amino acid sequence are shown in SEQ ID NO: 9 and 10, respectively.

Example 7. Structural analysis of the gene of aurone glycosyl transferase

pPh7GTa contained a 1750 bp gene, Ph7GTa, encoding a protein comprising 488 amino acids, and pPh7GTb contained a 1669 bp gene, Ph7GTb, encoding a protein comprising 476 amino acids. Using the deduced amino acid sequences obtained, they were compared with the AmGTI gene derived from Snapdragon obtained Example 3 and the pS.b UFGT1 gene derived from Scutellaria baicalensis. Accordingly, it was found that Ph7GTa had a 50% and 51% homology with AmGT1 and pS.b UFGT1, respectively. When compared with IS5a and IS10a derived from tobaccoes that are already reported to be genes having a high homology with pS.b UFGT1, they have exhibited homologies of 59% and 60%, respectively. Similarly, Ph7GTb had homologies of 59% and 56% with AmGT1 and pS.b UFGT1, respectively, and homologies of 88% and 86% with IS5a and IS10a derived from tabaccoes, respectively.

On the other hand, they only had a homology of about 20 to 25% with the gene of an enzyme (Tanaka et al. (1996) Plant Cell and Physiology 37: 711-716; Frutek D, Schiefelbein JW, Johnston F, Nelson Jr. OE (1988) Plant Molecular Biology 11: 473-481, Wise RP, Rohde W, Salamini F. (1990) Plant Molecular Biology 14: 277-279) that glycosilates the position 3 of flavonoids and the gene of an enzyme (WO 99/05287) that glycosilates the position 5 of flavonoids, and therefore, it was estimated that both of Ph7GTa and Ph7GTb are the genes of flavonoid-7-glycosyl transferase as are pS.b UFGT1 and AmGT1.

Example 8. Expression of Ph7GTa and Ph7TGTb cDNA in E. coli

The Ph7GTa gene was expressed using the pET System (Stratagene). First, in order to introduce NdeI and BamHI sites, the following 2 primers pETPh7GTa5' [5'-ATA ACT ACA TAT GGC TAT TCC CAC A-3' (SEQ ID NO: 11)] and

pETPh7GTa3' [5'-GAA CAG GAT CCT AAA AGG ACC T-3' (SEQ ID NO: 12)] were used in a PCR reaction.

The PCR reaction mixture was prepared in a total volume of 100 µl comprising 100 ng of pAmGT1, the 1 × the cloned Pfu DNA polymerase reaction buffer (Stratagene), 0.2 mM dNTPs, 0.5 pmol/µl each of the primers, and 5.0 Units of the cloned Pfu DNA polymerase. The reaction was carried out, after 45 seconds at 95°C, for 25 cycles with one cycle comprising 95°C for 45 seconds, 50°C for 45 seconds, and 72°C for 2 minutes, and was finally treated at 72°C for 10 minutes. The PCR product obtained was subcloned into the pCR2.1 TOPO vector (INVITROGEN). Some of the clones of the plasmid pTOPO-ETPh7GTa thus obtained were reacted using ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and the entire nucleotide sequences were confirmed using DNA Sequencer model 310 (Applied Biosystems). An about 1.7 Kb fragment obtained by digesting pTOPO-ETPh7GTa with NdeI and BamHI was ligated to the NdeI and BamHI sites of the pET-3a vector (Stratagene) to obtain plasmid pETPhGTa.

Using pETPhGTa, it was transformed into Epicurian Coli BL21 (DE3) (Stratagene).

For Ph7GTb also, in order to introduce NdeI and BamHI sites, the following 2 primers pETPh7GTb5' [5'-ATA ACT ACA TAT GGG TCA GCT CCA-3' (SEQ ID NO: 13)] and pETPh7GTb3' [5'-CTC GTA CCA TGG AAA ACT ATT CT-3' (SEQ ID NO: 14)] were used in a PCR reaction and then plasmid pETPhGTb was obtained.

Example 9. Measurement of the glycosyl transferase activity of Ph7GTa, Ph7GTb cDNA recombinant proteins

The transformants obtained in Example 8 were cultured, extracted and the enzymatic activity was measured as in Example 1. The enzymatic activity was measured using aureusidin as the substrate. The

enzymatic activity was measured as described in Example 1. For Ph7GTa and Ph7GTb, a peak was obtained that had the same retention time and the same spectrum as aureusidin 6-glycoside as a reaction product. For Ph7GTa also, one peak, that is estimated to be an aurone glycoside from the absorption spectrum, was obtained, and for Ph7GTb two such peaks were obtained.

The foregoing results revealed that Ph7GTa and Ph7GTb encode enzymes having an activity of glycosilating aureusidin.

Industrial Applicability

Using the gene expression products obtained in the present invention, it was possible to glycosilate aurones. This enabled a stable expression of aurones in plant cells.